

**Figure 4** Treatment of spasticity in autoimmune encephalomyelitis<sup>1</sup> with non-CB<sub>1</sub> receptor agonists. Forces (mean ± s.e.m.) required to flex individual spastic hindlimbs against a strain gauge after i.v. injection with either low-dose methanandamide (*n* = 9 limbs), JWH-133 (*n* = 9) or palmitoylethanolamide (*n* = 14). Asterisk, *P* < 0.05; two asterisks, *P* < 0.001 compared with baseline.

cannabinoids<sup>3</sup>. The use of selective CB<sub>2</sub> agonists may provide some symptomatic benefit without significant psychoactive effects. Furthermore, it may be possible to upregulate endogenous produced cannabinoids<sup>18</sup> to mediate therapeutic benefit. This CREAE model provides a means of evaluating and controlling the pathophysiology of spasticity in a chronic inflammatory environment relevant to the control of multiple sclerosis. □

**Methods**

**Induction of CREAE**

Biozzi ABH mice, bred at the Institute of Ophthalmology, were injected with 1 mg of mouse spinal cord homogenate emulsified in Freund's complete adjuvant on days 0 and 7 (ref. 1). Animals injected for CREAE, before the onset of acute phase CREAE<sup>1</sup> (usually occurring 15–20 days post inoculation (p.i.)) were used as normal CREAE controls. Paralysed CREAE animals were selected during the acute phase or first relapse (typically occurring 34–45 days p.i.), and remission animals used for the assessment of tremor and spasticity were used after the second or third relapse 40–80 days p.i.).

**Chemicals**

R(+)-WIN 55,212, S(-)-WIN 55,212, Δ<sup>9</sup>-THC, methanandamide and cannabidiol were purchased from RBI/Sigma (Poole, UK). Palmitoylethanolamide was purchased from Tocris Cookson Ltd (Bristol, UK). SR141716A (ref. 15) and SR144528 (ref. 16) were supplied by M. Mossé and F. Barth (Sanofi Research, Montpellier, France). JWH-133 (3-(1'-1'-dimethylbutyl)-1-deoxy-Δ<sup>8</sup>-THC) was synthesised as described<sup>19</sup>. All compounds were dissolved at 0.5 mg ml<sup>-1</sup> in ethanol containing 1 mg ml<sup>-1</sup> Tween 80 (Sigma). The ethanol was removed by vacuum drying, and samples were reconstituted with phosphate buffered saline to a concentration of 2 mg ml<sup>-1</sup>. Similar preparations without active drugs were used as vehicle controls. Suspensions (0.1 ml) were injected either i.v. or i.p. after CREAE induction.

**Assessment of Clinical Signs**

Spasticity and tremor were initially assessed by blinded analysis of video recordings. Digital images were sampled from video at 0.04 s. Signs of tail spasticity (flicking and curling) were assessed visually as being either present or absent. Spasticity was confirmed by assessing limb spasticity against a small purpose-built strain gauge. Limbs of animals without clinical evidence of spasticity (propensity to full extend the limb after tension on the leg) or the propensity to cross were not examined in drug studies. The analogue signal was amplified and digitally converted using an Amplicon card (Brighton, UK). This was captured using dacquire V10 software (D. Buckwell, MRC HMBU, Institute of Neurology) and analysed using Spike 2 software (Cambridge Electronic Design, UK). The hindlimbs were fully extended twice then moved to full flexion against the strain gauge. Each hindlimb was individually assessed by a blinded operator. The mean of 4–8 individual readings per limb was taken. Tremor frequency and severity were also recorded by holding the limb ~5 mm above the strain gauge. Tremor lead to the foot knocking the strain gauge. The strain gauge output was notch filtered at 50 Hz. The device had a resonance frequency of 95 Hz. The frequency of limb tremor was also confirmed using a lightweight unidirectional accelerometer (EGA XT-50, Entrain, UK) mounted over the foot.

**Statistical Analysis**

Results are expressed as means of individual feet or animals ± s.e.m. per group. The data were assessed using either a *t*-test, paired *t*-test for flexion data or nonparametric Mann-Whitney *U*-test using SigmaStat 2.0 software (Jandel Corp, San Rafael, California, USA).

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**Light acts directly on organs and cells in culture to set the vertebrate circadian clock**

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The expression of clock genes in vertebrates is widespread and not restricted to classical clock structures<sup>1,2</sup>. The expression of the *Clock* gene in zebrafish shows a strong circadian oscillation in many tissues *in vivo* and in culture, showing that endogenous oscillators exist in peripheral organs<sup>3</sup>. A defining feature of circadian clocks is that they can be set or entrained to local time, usually by the environmental light–dark cycle<sup>4,5</sup>. An important question is whether peripheral oscillators are entrained to local time by signals from central pacemakers such as the eyes or are themselves directly light-responsive. Here we show that the

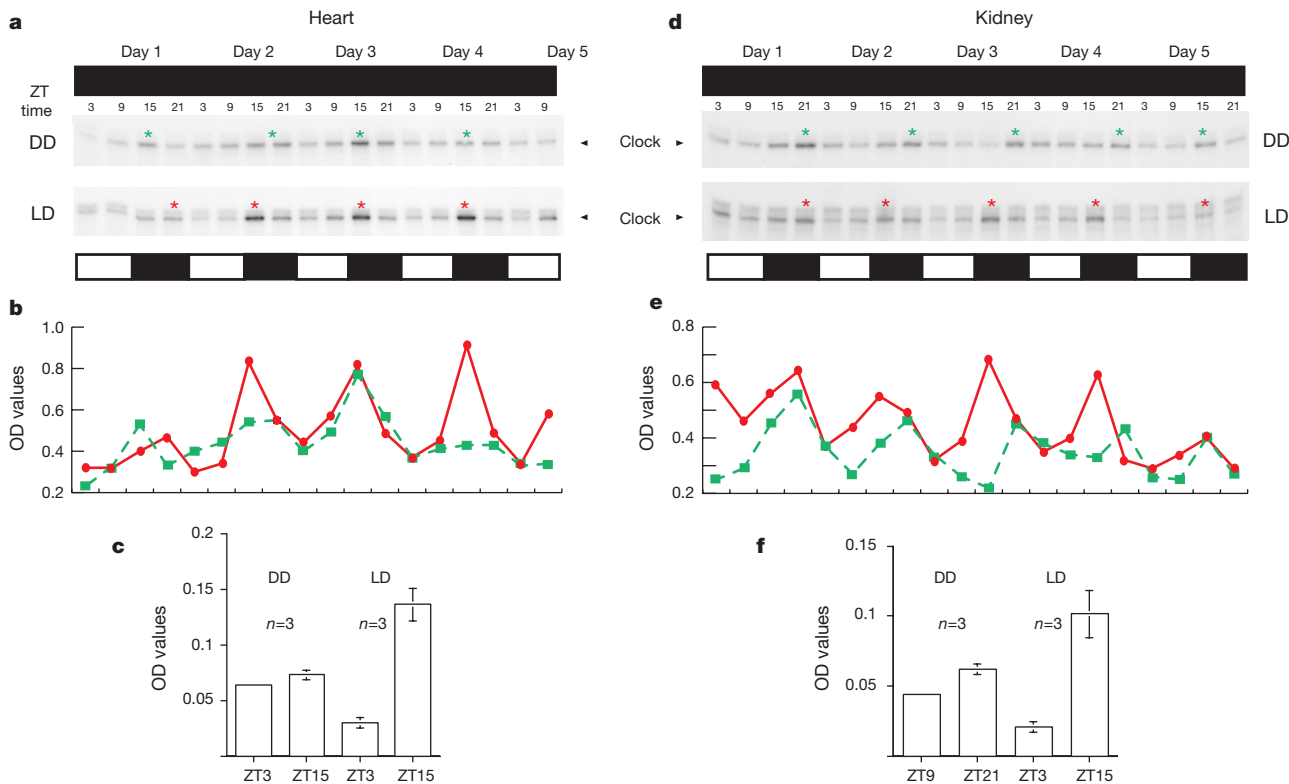
**peripheral organ clocks of zebrafish are set by light–dark cycles in culture. We also show that a zebrafish-derived cell line contains a circadian oscillator, which is also directly light entrained.**

The peripheral organs of zebrafish, in particular the heart and kidney, contain endogenous circadian oscillators, as measured by a rhythmic oscillation of *Clock* gene expression in culture<sup>3</sup>. To investigate how these clocks might be set to local time and the role played by light, we placed two groups of hearts and kidneys into culture, one group in constant darkness (DD) and another group on a light–dark cycle (LD) (Fig. 1). The cultured organs were exposed to a cycle of 14 hours of light and 10 hours of dark (14L:10D) over five days, matching exactly the cycle experienced by the fish before dissection. In DD, *Clock* levels continued to oscillate, although with a lower amplitude than that seen *in vivo*, peaking around zeitgeber time (ZT, where ZT 0 corresponds to ‘lights on’) 15 in the heart and ZT 21 in the kidney, as previously described<sup>3</sup> (Fig. 1a, b, d and e). In the LD cycle, however, the amplitude and robustness of *Clock* oscillation in the heart were greater than in the DD group (Fig. 1c). Rhythm amplitude increased similarly in the kidney (Fig. 1f) where, in addition, the phase of peak expression appears to be shifted from ZT 21 under DD to ZT 15 under the LD cycle (Fig. 1d and e), the phase of the peak *in vivo*. Thus, light appears to influence *Clock* oscillation directly in these organs in culture.

To determine whether light can entrain the peripheral clocks in these organs we placed both hearts and kidneys on a reversed light–dark cycle (DL) and studied the timing of *Clock* oscillation (Figs 2 and 3). Two groups of organs were dissected from the same fish population on a 14L:10D cycle, and immediately placed either on the same 14L:10D cycle (LD) or on a reversed, 10D:14L cycle (DL).

In hearts maintained on the LD cycle, *Clock* expression continued to peak at ZT 15. However, in hearts on the reversed, DL cycle, the first *Clock* peak was delayed by 12 h on day 2 in culture, at the equivalent of ZT 3 on the original LD cycle. From day 2 onwards the peak and trough values of *Clock* expression were reversed between the two groups (Figs 2a, b and 3d), consistent with the hearts being entrained to the reversed, DL cycle. The kidney shows the same reversal of peak expression, with stable re-entrainment shown by the beginning of day 3 of the DL cycle (Fig. 3a, b and d). An alternative explanation for the reversal of the *Clock* rhythm could be that light directly suppresses *Clock* expression. This is unlikely, as light in the zebrafish has no significant acute effect on *Clock* expression (data not shown), similar to the situation for the mouse *Clock* gene<sup>6–8</sup>. We also examined expression of another clock gene, *timeless*, under the same conditions as for *Clock*. Similarly to its mouse homologue<sup>9,10</sup>, zebrafish *timeless* neither oscillates nor responds to a light–dark cycle (Figs 2c and 3c). To show that the circadian oscillator is stably entrained to the reversed light cycle we repeated the experiment, placing the cultures into DD for the final two days. The oscillation should continue with the newly established phase, whereas in the case of an acute effect of light, the peak would return to the original phase seen in the animal. In the heart, the 12-h shift is conserved in DD, showing that the circadian clock is entrained to the new LD cycle (Fig. 2d). Light can, therefore, directly influence the circadian oscillator in peripheral tissues in culture and set the phase of the clock.

We wondered whether changes in temperature induced by the periodic lighting could be responsible for entraining these oscillations<sup>11–15</sup>. This seems unlikely, as the cultures were incubated in



**Figure 1** Exposure to light–dark cycles alters the pattern of *Clock* rhythmic expression in cultured zebrafish organs. **a**, Hearts were cultured for 5 days. At the indicated zeitgeber times (ZT) *Clock* messenger RNA was assayed by RPA (*Clock*-specific band indicated by arrowhead). Cultures were in constant darkness (DD) or a 14:10 light–dark cycle (LD). Asterisks, peaks of expression. **b**, Optical density (OD) measurements from **a**. The DD and LD values are green and red, respectively. **c**, Mean levels and s.e.m. at the trough (ZT 3) and peak (ZT 15) points, plotted for the fourth day in culture under DD and LD in additional

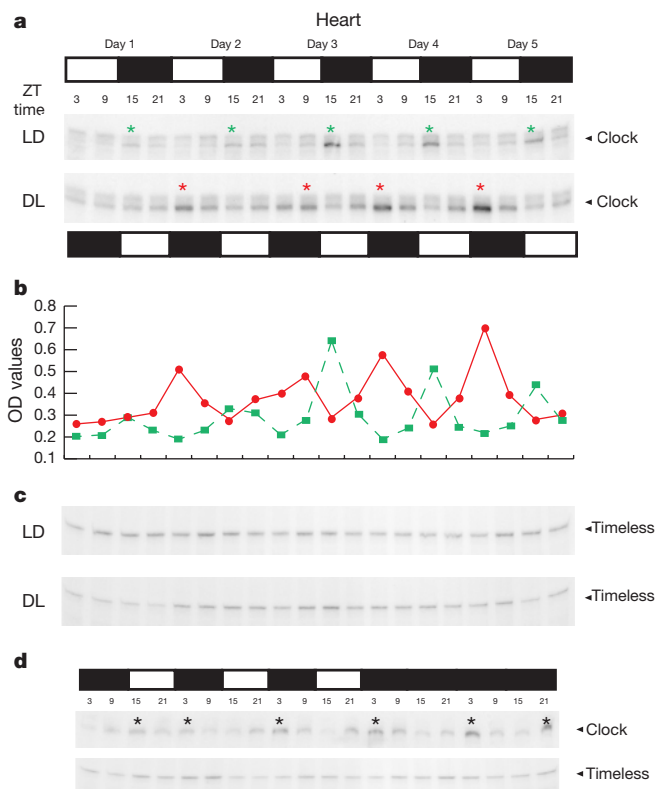
experiments. By unpaired Student’s *t*-test analysis there was a significant oscillation under LD ( $P < 0.005$ ) but not DD ( $P > 0.1$ ). **d–f**, Equivalent analysis for kidney. The ZT 21 peak of expression under DD is shifted to ZT 15 in LD. Peak and trough values were measured at ZT 3 and ZT 15 under LD and ZT 9 and ZT 21 under DD. Differences were significant for both LD ( $P < 0.01$ ) and DD ( $P < 0.01$ ). Equivalent loading was verified as described<sup>3</sup>.

large volume water baths where there were no detectable temperature changes. We also placed cultured tissue on a precise 2 °C temperature cycle in DD, mimicking the light–dark cycles previously used. The circadian clock failed to entrain to these temperature cycles, but maintained the pattern of expression expected in DD. Therefore, a 2 °C temperature cycle, which far exceeds any recorded differences during our experiments, is insufficient to entrain these zebrafish clocks. Given these results, we believe that light acts directly on the circadian clock.

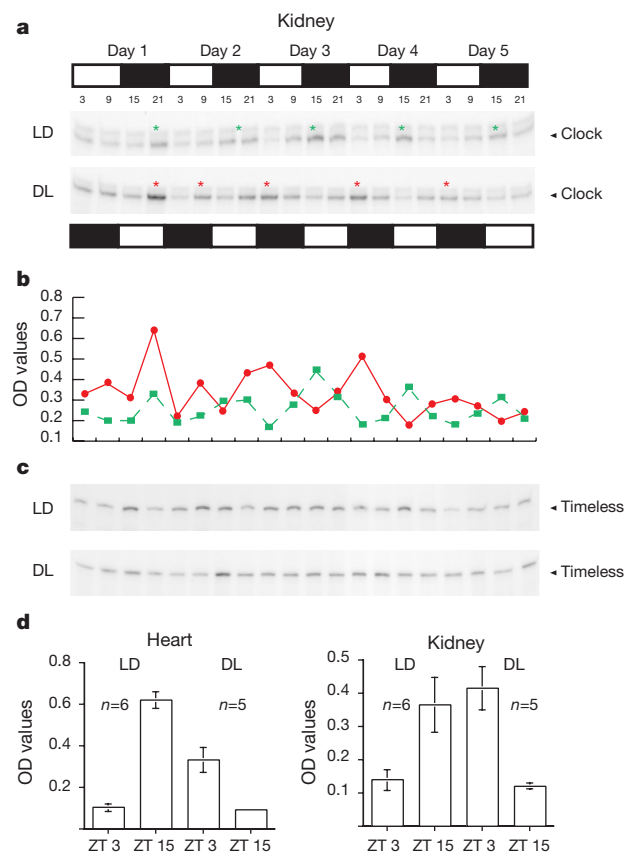
Evidence points to the existence of circadian oscillators even in immortalized cell lines<sup>16–18</sup>. Circadian oscillations in gene expression have been reported in cell lines derived from the suprachiasmatic nucleus (SCN) and can be induced in fibroblast and hepatoma cell lines by serum shock followed by starvation<sup>16,17</sup>. Therefore, we decided to examine a number of primary zebrafish cell lines<sup>19,20</sup> to see whether they naturally contained a circadian oscillator that could also be influenced by exposure to light–dark cycles. One zebrafish embryonic cell line, PAC-2, showed constant, elevated levels of *Clock* expression in DD (Fig. 4a). However, when these cells were placed on to a 14L:10D cycle, an oscillation in *Clock* levels was apparent on the first day of the regime (Fig. 4b), with a relatively broad and low amplitude peak spread between ZT 9 and ZT 15. By the second day of the light cycle, the peak of expression had consolidated at ZT 15 and the amplitude of the rhythm had increased. When these cells were returned to DD the oscillation

continued for two cycles, but with a reduced amplitude and broadening of expression (Fig. 4c). By the third day in DD the oscillation was no longer apparent. This continuation of rhythmicity in DD following exposure to an LD cycle supports the hypothesis that these cells contain a clock that is entrained by the light–dark cycle, rather than a driving or masking effect of light on *Clock* expression.

The light entrainability of the PAC-2 cells indicates that they may contain a functional circadian oscillator that operates under steady-state culture conditions. However, the absence of significant rhythmic *Clock* expression under DD indicates that the LD cycle either synchronizes single-cell oscillators with a random phase distribution or initiates circadian oscillation that was not functional before light exposure. Single-cell imaging of cells expressing a fluorescent reporter gene under the control of a clock-regulated promoter may help to resolve this issue. Our results indicate that this cell line contains the photopigments and functional signal transduction cascades necessary for the light signal to reach the clock mechanism. The identity of these photopigments is not yet clear. Action spectra, as well as functional experiments, will be required to determine whether cryptochrome pigments are used, as appears to be the case in *Drosophila*, or whether novel opsins are the critical phototransducing molecules<sup>13,21</sup>. In *Drosophila*, light-dependent entrainment of the circadian clock is thought to act through degradation of the Timeless protein as a consequence of sequestration by the cryptochrome photopigment<sup>22,23</sup>. It will therefore also be



**Figure 2** Clock-regulated rhythmic gene expression in the heart is entrained by reversed, DL cycles. **a**, RPA analysis of *Clock* transcript expression on LD or DL cycles for heart. Arrowheads, *Clock*-protected fragment; asterisks, times of peak expression. **b**, Optical density measurements from **a**. The rhythm of *Clock* expression is rapidly entrained to the reversed, DL cycle (red). For statistical analysis, see Fig. 3d. **c**, RPA analysis of zebrafish *timeless* expression in the same LD and DL samples. The non-oscillating, *timeless*-protected fragment is indicated. **d**, Hearts dissected from fish maintained in a normal, LD cycle were entrained to a reversed, DL cycle in L15 medium for 3 days in culture and then placed into DD for 2 days. In the upper gel, RPA of *Clock* expression shows that the re-entrained rhythm persists under DD. The lower gel shows non-oscillating *timeless* RPA analysis.



**Figure 3** Entrainment of the *Clock* rhythm in the kidney by reversed, DL cycles. **a–c**, RPA analysis of *Clock* and *timeless* transcript expression in cultured kidneys, equivalent to that shown in Fig. 2a–c. **d**, Mean levels of *Clock* expression with s.e.m. at the trough (ZT 3) and peak (ZT 15) points plotted for hearts and kidneys on the third day in culture. Underlined ZT values for DL refer to the original light–dark cycle. By unpaired Student’s *t*-test analysis differences between trough and peak expression were shown to be significant for both hearts and kidneys in both LD and DL conditions (hearts LD  $P < 0.0001$ , DL  $P < 0.005$ ; kidney LD  $P < 0.01$ , DL  $P < 0.005$ ).

of interest to study the effect of light on the expression of Timeless in our fish cell cultures.

The presence of a clock in an embryonic cell line may reflect the widespread presence of circadian clocks throughout the zebrafish body, where almost all tissues display rhythmic *Clock* expression (ref. 3 and unpublished data). Whether the clock is important during embryogenesis is unclear. However, rhythms in serotonin *N*-acetyltransferase (AANAT) and melatonin release have been described in embryos as early as 20–26 h after fertilization<sup>24,25</sup>. Our description of direct light entrainability of the oscillators in hearts, kidneys and PAC-2 cells indicates that this might be a general property of clocks in a wide range of peripheral tissues. This is an unexpected result in a vertebrate where entrainment of the circadian clock has been thought to rely on the presence of circadian photoreceptors in the eye or pineal gland. The organization of the circadian system in the fish, therefore, seems to resemble that reported for *Drosophila*<sup>26</sup>.

An important issue is the intensity of light that penetrates to particular organs. All of the experiments were performed initially in bright light of about 800 lux, but repeated with light intensities of approximately 200 lux with no apparent change in the speed of entrainment or amplitude of the resulting rhythm. In the context of the animal, it will be interesting to investigate whether other entraining signals such as melatonin or serum factors are involved<sup>16,27</sup>. Entrainment to the shifted LD cycle appears to occur rapidly: hearts and kidneys reach the new phase by the beginning of the second and third days of DL, respectively. PAC-2 cells show rhythmicity on the first day of an LD cycle. The rapidity of this response may reflect the fact that in a population of cells with randomly distributed circadian phases, some of the cells require only small phase shifts to reach a correct phase relationship to the

LD cycle. The speed of clock entrainment in fish organs is comparable to that seen in rodent retina *in vitro* and in isolated *Drosophila* appendages, which also contain clocks and are directly light entrainable<sup>26,28</sup>.

Our results add to the evidence that the circadian system in vertebrates exists as a decentralized collection of peripheral clocks. Individual organs have been previously shown to contain their own circadian oscillators<sup>3,29</sup>, and now each tissue appears capable not only of detecting environmental light signals, but also using that information to set the phase of the clocks they contain. □

## Methods

### Fish

Zebrafish were raised from our own stocks and kept at 29 °C. They were fed twice daily and maintained under a 14-h day, 10-h night cycle. Adult fish (4 months old) were killed by rapid immersion in chilled water and decapitation. Dissections were performed under PBS using microdissection tools and a dissection microscope.

### RNA analysis

RNA was extracted from zebrafish tissues and PAC-2 cell cultures using TRIzol reagent (Gibco BRL). We used a miniaturized RNase protection assay (RPA) using a zebrafish *Clock* complementary DNA-derived probe to assay *Clock* transcript expression as described<sup>3</sup>. A *timeless* probe for RPA was generated from zebrafish expressed sequence tag (EST) clone fa95g04. The 450-nucleotide probe corresponds to the carboxy-terminal region of mouse *timeless* between amino acids 921 and 1054, with which it shares 61% amino-acid similarity. Each point was prepared from a pool of six hearts or kidneys or one confluent 25-cm<sup>2</sup> flask of PAC-2 cells. RPA autoradiographs were scanned on an imaging densitometer (Biorad) and quantified using Molecular Analyst software (Biorad).

### In vitro organ and cell culture

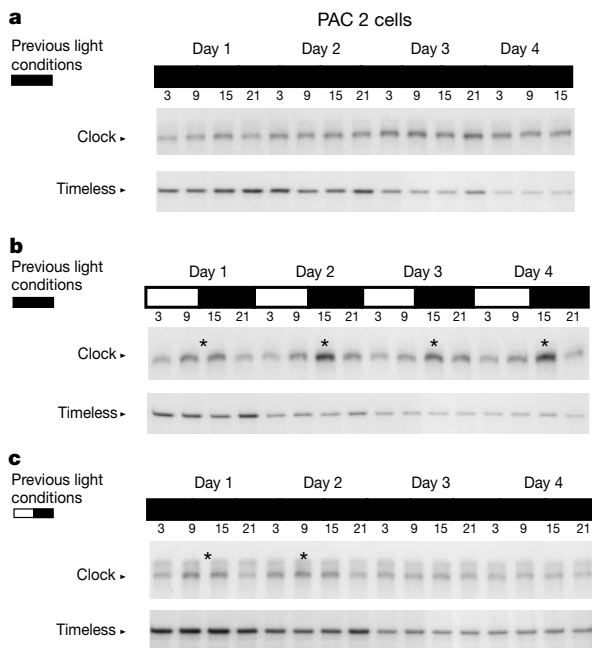
For *in vitro* organ cultures, freshly dissected tissue was placed in L15 medium supplemented with 15% fetal calf serum, 2 mM glutamine, gentamycin, streptomycin and penicillin. Organs were dissected from fish between ZT 9 and ZT 12 and placed directly into culture at 25 °C and atmospheric CO<sub>2</sub> concentration. A subline derived from the zebrafish embryonic cell line PAC-2 (ref. 19) was cultured as described above. Every 5 days, cells were trypsinized and replated at a 1:4 dilution. Cell stocks were maintained in non-illuminated incubators.

### Lighting and temperature control

Culture flasks were completely immersed in a 60-litre, thermostatically controlled, circulating water bath. These cultures were illuminated using a tungsten light source connected to a programmable timer and a rheostat. The temperatures of cultures in the water bath were monitored using a thermocouple immersed in the culture medium, and light intensities were measured using a light meter (Gossen).

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**Figure 4** A zebrafish cell line contains a light-entrainable circadian clock. **a**, In cultures of the embryonic cell line PAC-2 maintained for 4 days in constant darkness no significant oscillation in *Clock* expression is detected. **b**, PAC-2 cultures previously maintained under DD were switched to an LD cycle (14 h light:10 h dark). A robust rhythm of *Clock* expression is first detected on day 1 and persists for the duration of the experiment. The peaks, around ZT 15, are indicated by asterisks. **c**, Cultures previously exposed for 5 days to a 14:10 LD cycle were returned to DD and *Clock* expression was monitored. The same pattern of rhythmic *Clock* expression persists for 2 days, but gradually declines during the last 2 days of the experiment. Below each *Clock* RPA analysis panel is shown the results of RPA for *timeless* expression in the same samples. The rhythm of expression visible for *Clock* is absent for *timeless*.

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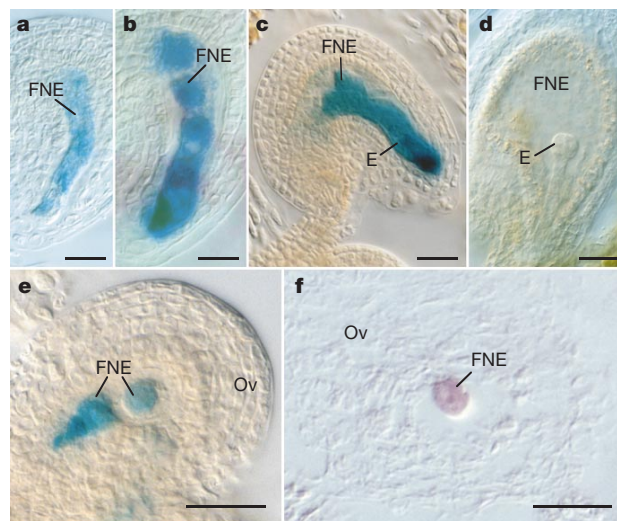
**Delayed activation of the paternal genome during seed development**

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Little is known about the timing of the maternal-to-zygotic transition during seed development in flowering plants. Because plant embryos can develop from somatic cells or microspores<sup>1</sup>, maternal contributions are not considered to be crucial in early embryogenesis<sup>2</sup>. Early-acting embryo-lethal mutants in *Arabidopsis*, including *emb30/gnom* which affects the first zygotic division<sup>3,4</sup>, have fuelled the perception that both maternal and paternal genomes are active immediately after fertilization. Here we show that none of the paternally inherited alleles of 20 loci that we tested is expressed during early seed development in *Arabidopsis*. For genes that are expressed at later stages, the paternally inherited allele becomes active three to four days after fertilization. The genes that we tested are involved in various processes and distributed throughout the genome, indicating that most, if not all, of the paternal genome may be initially silenced. Our findings are corroborated by genetic studies showing that *emb30/gnom* has a maternal-effect phenotype that is paternally rescuable in addition to its zygotic lethality. Thus, contrary to previous interpretations, early embryo and endosperm development are mainly under maternal control.

In flowering plants, double fertilization involves two sperm cells: one fuses with the egg cell to form a diploid zygote; the second fuses



**Figure 1** Silencing of paternally inherited genes during seed development in *Arabidopsis*. **a**, If an ET2612 female is crossed to a wild-type male, *GUS* expression is detected in the free nuclear endosperm 12 h.a.p. **b,c**, F<sub>1</sub> seeds of the same cross show more intense *GUS* expression in embryo and endosperm 48 h.a.p. **d**, If ET2612 is crossed as the male to wild-type plants, *GUS* expression is not detectable in embryo and free nuclear endosperm 48 h.a.p. **e**, Transverse optical section through a seed of a self-pollinated ET2612 plant 48 h.a.p. **f**, *In situ* hybridization to mRNA of the putative basal transcription factor tagged in ET2612; the pattern of mRNA and *GUS* expression are identical 48 h.a.p. E, embryo; FNE, free nuclear endosperm; Ov, ovule. Scale bars, 17 μm (**a,b**); 23 μm (**c**); 45 μm (**d**); 40 μm (**f**).

with the binucleated central cell to give rise to the triploid primary endosperm nucleus<sup>5</sup>. Double fertilization triggers rapid proliferation of the endosperm and slow cell divisions of the zygote, which usually undergoes an asymmetrical division<sup>6</sup>. In *Arabidopsis*, as in most plant species, the primary endosperm nucleus undergoes divisions without cytokinesis, giving rise to a syncytium that eventually cellularizes<sup>7</sup>. In contrast to animals<sup>8–10</sup>, the timing of transcriptional activation of the genome in the plant embryo and endosperm has not been intensively studied<sup>11</sup>. The identification of a large group of early-acting embryo-lethal *Arabidopsis* mutants that segregate as sporophytic recessive traits<sup>12</sup> suggested that the activation of the zygotic genome occurs before the first division of the zygote; however, a spatial and temporal pattern for zygotic genome activation has not yet been determined.

We generated a library of enhancer detector and gene trap lines (transposants) that harbour *Ds* elements with a *uidA* reporter gene encoding β-glucuronidase (*GUS*) by using the system of Sundaresan *et al.*<sup>13</sup>. Screening for genes that act during ovule and early seed development in *Arabidopsis* (U.G. *et al.* unpublished data), we identified 19 transposants that show *GUS* expression in the developing embryo and/or endosperm after fertilization. *GUS* is expressed in the egg and/or central cell and persists for several rounds of cell division in either one or both fertilization products. To determine whether *GUS* expression was the result of transcription from one or both parental alleles in each of these lines, we performed reciprocal crosses between wild-type plants and the 19 transposants. When wild-type plants were used as male parents, the resulting F<sub>1</sub> seeds showed *GUS* expression in a pattern identical to the one found in developing seeds resulting from self-pollination in all 19 lines (Fig. 1a–c). In contrast, if the transposants were used as male parents, *GUS* expression was absent from all F<sub>1</sub> seeds and remained undetectable up to 80 hours after pollination (h.a.p.) (Fig. 1d). This indicates that the paternally inherited allele may not be expressed during early stages of embryo and endosperm development. To verify whether the pattern of *GUS* expression truly reflects the expression of genes neighbouring the insertion, we performed

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